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13. ABSTRACT (Maximum 200 Words)

The *Saccharomyces cerevisiae* Rad9 checkpoint protein contains only one set of known domains - the BRCT domains. Originally identified in the C-terminus of the human breast cancer susceptibility gene *BRCA1*, these domains occur frequently in proteins involved in recognizing and/or repairing DNA damage. To better understand the role of the two C-terminal BRCT domains in Rad9, various deletion constructs were created and either overexpressed or endogenously expressed in *rad9Δ* strains. These strains were assayed for their ability to repair different forms of DNA damage and for their ability to arrest in response to such damage. Other indicators, such as downstream phosphorylation of Rad53, were also analyzed as signs of activation of the rad9 deletion constructs. Our results show that the BRCT domains are essential for Rad9's checkpoint functions. These domains act to increase the local concentration of Rad9 both by stabilizing the protein and by dimerizing the protein.

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INTRODUCTION

Rad9 is one of several checkpoint proteins operating in the budding yeast *S. cerevisiae*. The molecular roles of Rad9 are now coming to light. In a current model, Rad9 interacts with proteins that directly recognize DNA damage to form a complex that functions to propagate an arrest response by activating downstream protein kinases Rad53 and Chk1 (Navas et al., 1996; Sanchez et al., 1999). Directly binding to DNA damage are the Mec1-Ddc2 and Rad24-Rad17-Mec3-Ddc1 groups of checkpoint proteins (Melo et al., 2001; Kondo et al., 2001). Presence of these proteins at damage leads to Mec1-dependent hyperphosphorylation of Rad9 – a protein modification required for Rad9 interaction with the downstream protein kinase Rad53 (Schwartz et al., 2002; Sun et al., 1998; Emili, 1998; Vialard et al., 1998). Other have shown that Rad9 also dimerizes under damaging conditions, and the combined effect of Rad9 phosphorylation and its dimerization brings two Rad9-associated Rad53 molecules in close enough proximity to induce their autophorphorylation (Soulier & Lowndes, 1999; Gilbert et al., 2001). Additionally, Rad9 activates the Chk1 protein kinase, though the molecular details leading to Chk1 activation are unclear (Sanchez et al., 1999).

Key molecular questions in Rad9 biology include: 1) how does Rad9 get to DNA damage, 2) how does Mec1 recognize Rad9, 3) what role does the Rad24-Rad17-Mec2-Ddc1 complex play in Rad9 phosphorylation, 4) does Rad9 phosphorylation induce dimerization, and, 5) what Rad9 modifications are necessary for Chk1 activation? Answers to these questions might be found in understanding the role of Rad9's only known functional domains, the BRCT (BRCA1 carboxyl terminus) repeats.

First identified in the C-terminus of the human breast cancer susceptibility protein BRCA1, the BRCT domains occur frequently in proteins involved in DNA damage response (Koonin et al., 1996; Bork et al., 1997; Callebaut & Mornon, 1997). More than 50 BRCT-containing proteins have now been identified in essentially all organisms (Huyton et al., 2000). Examples include the fission yeast replication checkpoint protein Rad4, the mammalian DNA repair protein XRCC1, the budding yeast DNA polymerase subunit Dpb11, and the bacterial DNA ligase LigA. Though the exact molecular functions of these proteins are diverse, they all play some role in responding to DNA damage. Moreover, all BRCT-containing proteins possess other large domains with distinct activities, possibly suggesting that the BRCTs play a role in signal transduction, linking together proteins that process or control DNA structure (Bork et al., 1997). Since all BRCT-containing proteins are involved in some aspect of DNA metabolism, some DNA function is implied.

The roles of the BRCT domains in any protein have yet to be fully characterized. The majority of the literature suggests that the BRCT domains mediate protein-protein interactions, including homotypic dimerization of proteins via their BRCT domains and other heterotypic BRCT-nonBRCT interactions, while other studies implicate the BRCT domains in DNA binding. For example, the XRCC1 protein, which functions in the base excision repair pathway as a scaffolding protein, contains two BRCT domains that each interact with the BRCT domains within the proteins DNA ligase III and poly ADP ribose polymerase (Caldecott et al., 1995; Nash et al., 1997; Taylor et al., 1998; Masson et al., 1998). BRCT dimerizaton is supported by XRCC1 BRCT crystal structure analysis that reveals a hydrophobic, autonomously folded domain with a globular structure consisting

of a four-stranded parallel β-sheet core surrounded by three α-helices (Zhang et al., 1998). Moreover, two crystal structures of an XRCC1 BRCT domain suggest two different packing arrangements – it has been suggested one for inter-BRCT associations between two proteins and one for intra-BRCT associations between domains within the same protein (Huyton et al., 2000). Other work indicates that the BRCT domains in XRCC1 and in other proteins function to bind DNA strand breaks and DNA termini. XRCC1, the topoisomerase binding protein (TopBP1), and BRCA1 each bind to DNA via their BRCT domains (Yamane et al., 2000 (BRCA1 & XRCC1); Yamane & Tsuruo, 1999). Of note, BRCT-mediated binding occurred at both single- and double-stranded DNA ends and nicked circular DNA, but not to circular double-stranded DNA, and binding was not dependent on DNA sequence (Yamane & Tsuruo, 1999; Yamane et al., 2000 (BRCA1 & XRCC1)). Furthermore, protein binding conferred exonuclease-resistance to the ends of the DNA fragments bound (Yamane & Tsuruo, 1999; Yamane et al., 2000 (BRCA1 & XRCC1).

Because Rad9 possesses two BRCT domains, these domains may function in homodimer formation, heterodimer formation, and/or direct DNA binding, as has been demonstrated for other BRCT-containing proteins. Indeed, Rad9 has been shown to homodimerize through its BRCT domains, particularly in response to DNA damage (Soulier & Lowndes, 1999). Specific point mutations of Rad9's BRCT domains (rad9-BRCT^{MutF+W}) does not allow Mec1-dependent hyperphosphorylation of Rad9 after DNA damage, nor is Rad53 activated, which is necessary to elicit a cellular arrest response (Soulier & Lowndes, 1999). Moreover, this Rad9 BRCT mutant protein is abrogated in its ability to homodimerize, suggesting that Rad9 homodimerization via the BRCT domains contributes to Rad9 phosphorylation and activation of Rad53 and, therefore, to checkpoint arrest and repair (Soulier & Lowndes, 1999). The possibility that Rad9's BRCT domains affect its association or heterodimerization with another protein needed for checkpoint activation still exists, as does the possibility that Rad9 can directly recognize and bind to DNA damage via its BRCT domains. Also unresolved is whether Mec1-dependent Rad9 hyperphosphorylation is necessary to induce Rad9 homodimerization or whether homodimerization precedes Mec1-dependent hyperphosphorylation. More direct and rigorous tests of Rad9 homodimerization are provided herein.

To better characterize the BRCT domains in Rad9, we investigated the effects of specific Rad9 mutations. We show that removal of the BRCT domains (rad9ΔBRCTs) completely abrogates Rad9's ability to activate Rad53 and Chk1, in accord with disruption of arrest and DNA repair in response to damage. However, complete function could be restored by either overexpressing the *rad9ΔBRCTs* allele or by forcing the protein to artificially dimerize by fusion to the GST protein (rad9ΔBRCTs-GST). *In vivo* analysis of cell cycle arrest (S- and G_2/M phases) and DNA damage resistance (UV and MMS) was performed, along with biochemical analysis of Rad9 homodimerization and activation of the downstream kinases, Rad53 and Chk1. Preliminary co-immunoprecipitation results suggest that dimerization of Rad9 is not rate-limiting for function, as was previously thought. Additional genetic analysis of point mutations within Rad9's BRCT domains indicates that the BRCT repeats may affect proper protein localization or may negatively regulate Rad9 function. In total, our data demonstrates that the BRCT domains are predominantly required for Rad9 homodimer formation and

protein stabilization, and we deduce that the dimerized state is required for the local concentration of Rad9 at sites of damage to facilitate Rad9-dependent reactions. In addition, Rad9 may be subject to negative regulation, which includes the possibility of regulation of protein localization, under certain cellular conditions to prevent checkpoint activation.

BODY

Expression of rad9ΔBRCTs results in DNA damage sensitivity.

To assess the contribution of the BRCT domains to Rad9 DNA damage checkpoint functions, we deleted these domains and analyzed the impact of their loss on cell phenotypes by expressing the rad9ΔBRCTs protein in cells subjected to DNA damage (Figure 3-1a). DNA damage of cells included either UV irradiation or inactivation of proteins encoded by the temperature-sensitive *cdc13-1* allele, which results in loss of Cdc13 at telomere ends and leaves them prone to exonucleolytic degradation (Lydall & Weinert, 1995). Whereas wild-type *RAD9* expression resulted in a robust cell cycle arrest after *cdc13-1*-induced damage, expression of the *rad9ΔBRCTs* truncation under the *RAD9* promoter yielded no such arrest response (Figure 3-1b). Likewise, the wild-type *RAD9* strain was able to maintain high cell viability after UV-damage treatment, but the *rad9ΔBRCTs* strain again exhibited a null response for repair of UV-induced damage (Figure 3-1c). Curiously, the abundance of rad9ΔBRCTs protein is severely diminished in comparison with wild-type Rad9 protein levels, though this has no apparent bearing on rad9ΔBRCTs functions (discussion follows). Therefore, under control of the *RAD9* promoter, Rad9's BRCT domains are necessary to initiate a cell cycle checkpoint arrest in the presence of damage and for repair UV-induced DNA lesions.

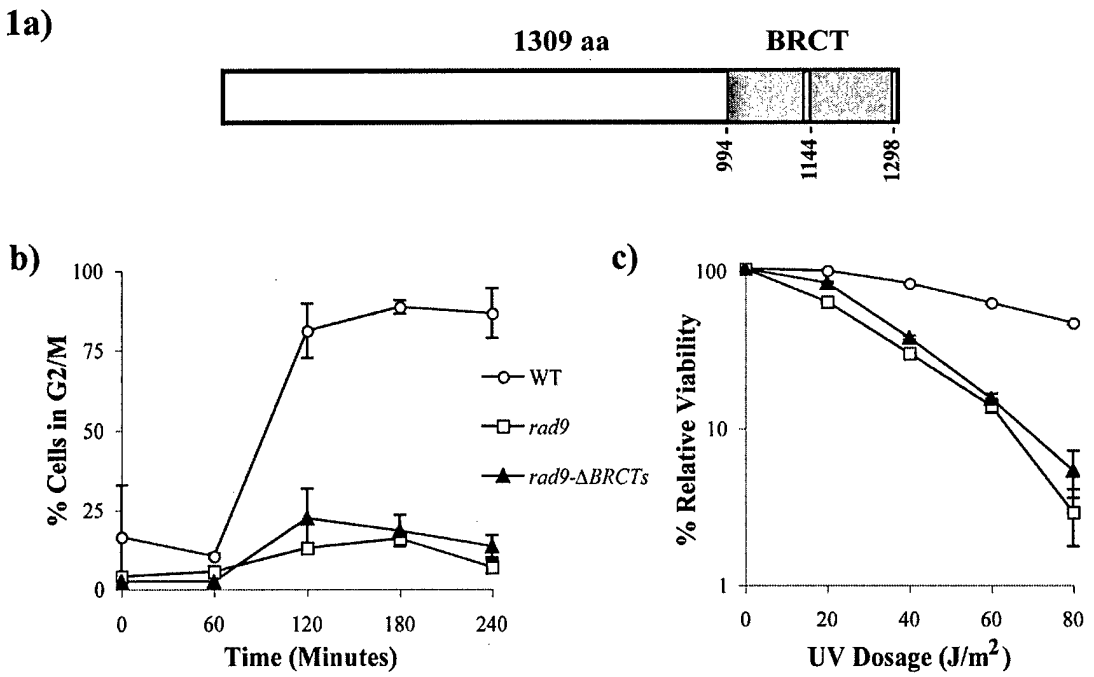

Figure 3-1. A map of the Rad9 protein and phenotypic effects of deleting the BRCT domains. (*a*) A map of the Rad9 protein sequence showing locations of the BRCT domains. (*b*) An assay for cell cycle arrest function of the *rad9ΔBRCTs* allele. Mid-logarithmic cells were synchronized in G_1 with α-factor at 23°C. Cells were then released at the restrictive temperature (36°C) to inactivate *cdc13-1* and induce DNA damage. Cells were fixed at various time points and subsequently scored for cell cycle position by analysis of DAPI-stained nuclei with fluorescent microscopy. (*c*) Assessing UV sensitivity of the *rad9ΔBRCTs* allele. Mid-logarithmic cells were plated to solid media, exposed to various doses of UV irradiation, and allowed to grow overnight at 23°C. Cells were then scored the following day for the ability to form microcolonies, indicative of UV resistance. For the cellular assays, each strain was tested in duplicate. The average of the duplicates is shown above, with error bars indicating the standard deviation from the mean.

Overexpression of rad9ΔBRCTs confers DNA damage resistance.

Using a different means to determine the contribution of the BRCT domains to Rad9 functions, we compared the effects of overexpressing Rad9 versus two Rad9 fragments - one consisting of Rad9 lacking the BRCT domains (rad9ΔBRCTs) and the other comprised solely of the BRCT repeats (BRCTs). To this end, we created plasmid overexpression constructs, where our various *RAD9* alleles were placed under the control of the Gal promoter. When placed in galactose-containing media, the overexpression constructs produced a dramatic increase in protein levels as compared to levels of Rad9 protein expression under control of the normal promoter (Fig. 2a). We found the wild-type Rad9 protein to be overexpressed over 500-fold above normal Rad9 protein levels, while the rad9ΔBRCTs protein was subject to a much more moderate 25-fold induction above wild-type Rad9. The diminished overexpression of the rad9ΔBRCTs protein suggests that the BRCT domains may play a role in stabilizing wild-type Rad9 proteins.

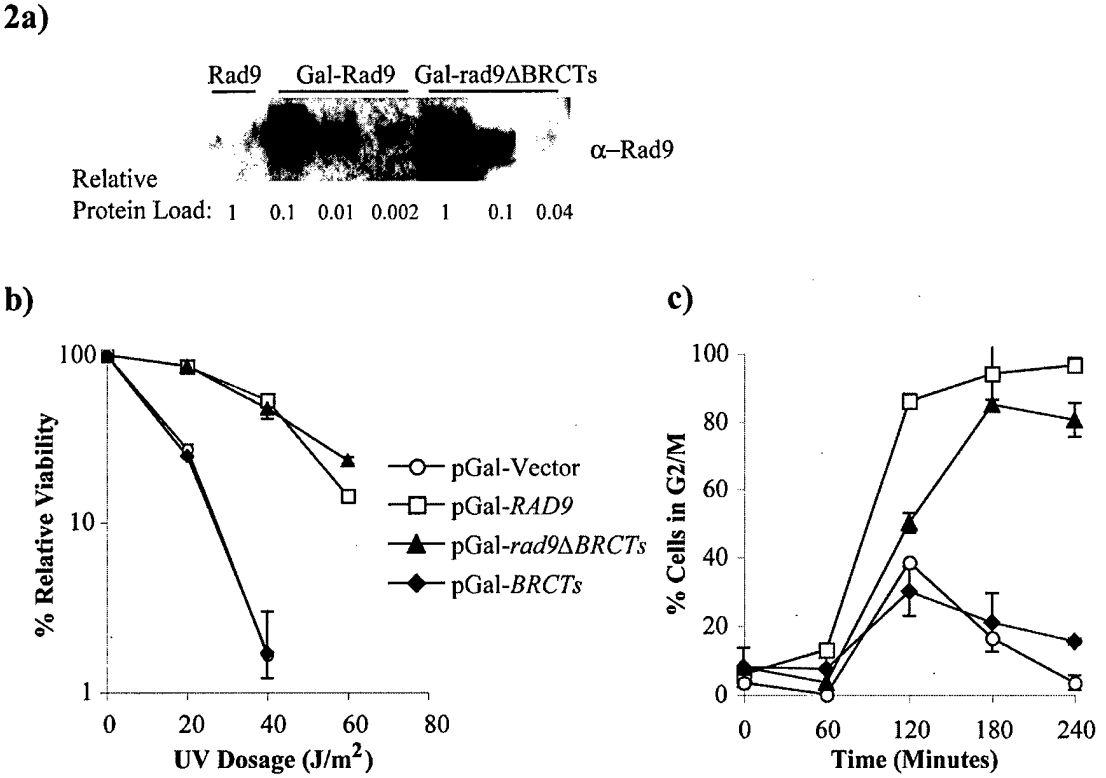

Figure 3-2. Overexpression levels of the rad9ΔBRCTs protein and phenotypic analysis. (*a*) Mid-logarithmic cells were grown in the presence of 3% galactose for 3 hours before being lysed in 20% TCA. For each sample, the same density of cells was lysed for protein analysis. Serial dilutions of proteins are shown above, with the relative loads indicated underneath each lane. An α-Rad9 antibody (gift from D. Sterns) was used for detection of Rad9 protein. (*b*) For determination of UV sensitivity, mid-logarithmic cells were plated to minimal media, subjected to UV irradiation, and grown overnight. Cells were scored the following day for microcolony formation. (*c*) To determine cell cycle arrest proficiency, cells were arrested in G_1 with α-factor and then released at the restrictive temperature (36°) to induce *cdc13-1* damage. Cells were fixed at various time points and scored for cell cycle position using fluorescent microscopy of DAPI-stained nuclei. For (*b*) and (*c*), each strain was assayed in duplicate. The average of the duplicates is shown with error bars representing standard deviations from the mean.

To analyze the contribution of the BRCT domains to Rad9-mediated damage resistance in strains overexpressing the truncated rad9ΔBRCTs protein, strains were again assayed for their ability to withstand DNA damage caused by UV insult and for the ability to arrest at the G_2/M checkpoint in response to *cdc13-1*-induced damage. In sharp contrast to what we found when expressing rad9ΔBRCTs from the *RAD9* promoter, overexpressing the rad9ΔBRCTs protein in *rad9*⁻ cells fully restored the resistance to UV

damage (Figure 3-2b) and almost fully restored the DNA damage-dependent cell cycle arrest (Figure 3-2c). Overexpression of the BRCTs alone, however, had no effect, as this strain exhibited a *rad9-* phenotype. Because the overexpressed rad9ΔBRCTs construct produced nearly wild-type phenotypic responses, these data suggest that the BRCT repeats are not required *per se* for cell cycle arrest in the presence of damage or repair of UV damage when the Rad9 N-terminus is present at increased levels 25 times that of normal levels.

Overexpression of the BRCTs alone does not cause a dominant-negative effect.

Although overexpression of just the BRCT domains produced no phenotype in a *rad9*⁻ background, we hypothesized that overexpression of the BRCTs in a *RAD9*⁺ background may result in a dominant-negative phenotype because Rad9 is known to homodimerize via its BRCT domains, and it is hypothesized that dimerization is required for checkpoint function (Soulier & Lowndes, 1999). By this reasoning, we predicted that a wild-type *RAD9*⁺ strain overexpressing the BRCTs fragment would become sensitized to DNA damage insult because the free BRCT repeats would dimerize with the BRCT domains in the endogenous Rad9 proteins to render them non-functional. To assess any dominant-negative effects, we independently overexpressed the BRCTs protein and the rad9ΔBRCTs protein in a wild-type *RAD9*⁺ background and then subjected the cells to DNA damage. Interestingly, all strains showed a wild-type response for both arrest proficiency and UV resistance, and, therefore, caused no dominant-negative effect (data not shown).

Overexpression of Rad9 or rad9ΔBRCTs does not cause constitutive arrest.

To determine if high protein levels of Rad9 or rad9ΔBRCTs could induce a constitutive arrest response in the absence of DNA damage, the proteins were overexpressed in a *CDC13*⁺ strain. Cells were fixed over time and DAPI-stained cell nuclei were analyzed for cell cycle position. No such constitutive arrest was seen when overexpressing Rad9 or rad9ΔBRCTs (data not shown), leading us to infer that a Rad9-activating event must precede arrest – presumably recruitment of other checkpoint proteins (e.g., Mec1, Ddc2, Rad17, Rad24, etc.) to sites of DNA damage that then, in turn, leads to Rad9 phosphorylation.

Restoration of wild-type function of rad9ΔBRCTs by forced dimerization.

Given that Rad9 was previously shown to homodimerize via its BRCT domains, and based on our data that overexpression of the rad9ΔBRCTs protein obviated the need for the BRCTs in arrest and UV damage responses, we reasoned that the BRCT domains might be necessary to increase local concentrations of Rad9 for function by homodimerization. Our data support a model whereby under normal levels of expression, a *RAD9* allele lacking the BRCT repeats does not allow for homodimerization and, consequently, insufficient local concentration of Rad9 is achieved for checkpoint function, accounting for the null phenotype of the *rad9ΔBRCTs* strain. Overexpression of rad9ΔBRCTs, however, circumvents the need for local concentration through homodimerization by increasing protein concentrations enough within the cell to carry out checkpoint functions.

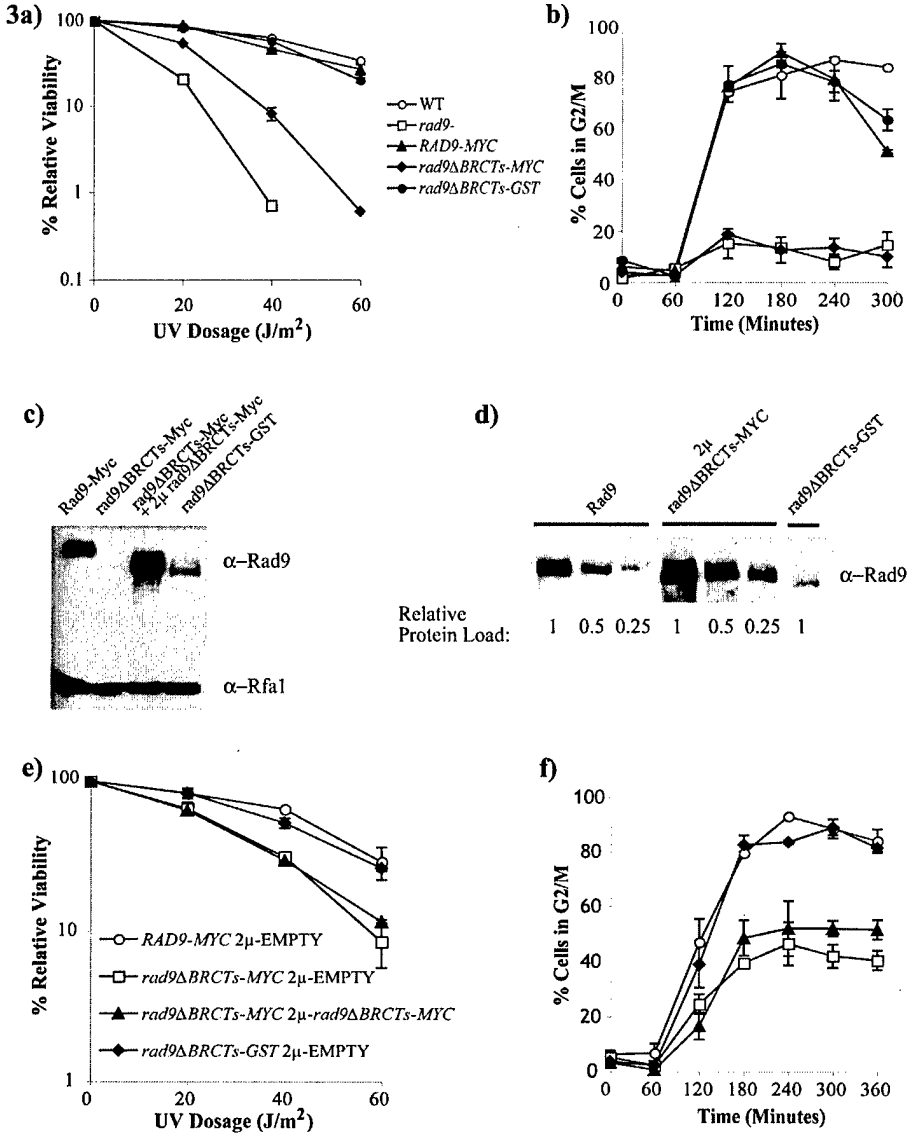

Figure 3-3. Characterization of strains containing non-dimerizing rad9ΔBRCTs-Myc and dimerizing rad9ΔBRCTs-GST proteins. (*a*) UV sensitivity, (*b*) arrest proficiency, (*c*) and protein expression levels of strains containing Rad9 and truncated proteins under normal levels of expression. (*d*) Comparison of protein expression levels between endogenously expressed Rad9, truncated rad9ΔBRCTs-GST, and the *rad9ΔBRCTs-MYC* strain carrying a 2μ multi-copy plasmid containing the *rad9ΔBRCTs-MYC* sequence. Serial dilutions of protein are shown with the relative loads indicated underneath. The same volume of cells was assayed for protein extraction. (*e*) UV sensitivity and (*f*) arrest proficiency of strains containing either a 2μ plasmid empty vector or the 2μ *rad9ΔBRCTs-MYC* plasmid. Cell cycle assays were performed as previously described. Each strain was assayed in duplicate. The average value of the duplicates is shown with error bars representing the standard deviation from the mean.

To determine if dimerization via the BRCT domains is necessary for Rad9 function under normal expression levels of protein, we artificially forced dimerization of rad9ΔBRCTs to see if this would restore function. To force dimerization, we used the GST protein, which has been reported in the literature to form dimers and tetramers (Ladbury *et al.*, 1995; Walker *et al.*, 1993; Ji *et al.*, 1992; Parker *et al.,* 1990). Moreover, the GST protein has been proven to be a useful substitute for the oligomerization domains of heterologous proteins (Maru *et al.*, 1996; Riley *et al.*, 1996; Maru, 2000), and can also be used to dimerize a protein of interest that does not oligomerize under normal conditions (Tudyka & Skerra, 1997).

When the GST protein was placed at the C-terminal end of the rad9ΔBRCTs protein (rad9ΔBRCTs-GST) expressed from the *RAD9* promoter, wild-type function for arrest and UV resistance was restored (Fig. 3-3a, b). Conversely, placement of the Myc epitope at the C-terminal end of this protein (rad9ΔBRCTs-Myc) did not restore either UV resistance or arrest function, as these strains demonstrated nearly null phenotypes under normal levels of expression.

In looking at protein expression levels, we found the rad9ΔBRCTs-Myc and the rad9ΔBRCTs-GST proteins to be expressed at levels less than that of wild-type Rad9, with rad9ΔBRCTs-Myc expression being even less than that of rad9ΔBRCTs-GST (Fig. 3-3c, d). Therefore, to determine whether the null phenotype for arrest and the hypomorphic UV-resistance phenotype associated with the *rad9ΔBRCTs-MYC* strain were due to low protein levels or the lack of BRCT domain functions, we boosted rad9ΔBRCTs-Myc expression by placing it in a multi-copy 2μ plasmid. While the multi-copy plasmid restored rad9ΔBRCTs-Myc protein expression to levels approximately twice that of wild-type Rad9 (Figure 3-3d), the null phenotypes were not complemented by the increase in protein dosage (Figure 3-3e, f). (Of note, the arrest response of the *rad9ΔBRCTs-MYC* 2μ-*rad9ΔBRCTs-MYC* strain does increase, but it also increases in the *rad9ΔBRCTs-MYC* strain carrying the 2μ empty vector. We attribute this apparent increase in arrest to different growth conditions – minimal media versus rich media – that might affect cell cycle kinetics.) These data indirectly indicate that dimerization of the Rad9 N-terminus, whether via the C-terminal BRCT domains or another dimerizing domain such as the GST protein, is critical for Rad9 checkpoint functions. Moreover, because the dimerizing GST protein can completely complement loss of the BRCT domains, this suggests that the sole role of the BRCTs in DNA damage arrest and UV resistance is to support dimerization of the Rad9 N-terminus.

Direct tests to confirm rad9ΔBRCTs-GST constitutively dimerizes.

We infer the *rad9ΔBRCTs-GST* strain to be wild-type for cell cycle arrest and UV repair responses because the GST protein is forcing dimerization of the Rad9 N-termini – a state necessary for interaction with the downstream checkpoint component Rad53 and perhaps also with Chk1. To verify dimerization of rad9ΔBRCTs proteins caused by GST homodimer interactions, we created strains with differentially tagged rad9ΔBRCT-GST proteins - *rad9ΔBRCT-GST-MYC* and *rad9ΔBRCT-GST-HA* – so that co-immunoprecipitations could be done. The dually tagged *rad9ΔBRCT-GST* strain, along with dually tagged positive and negative control strains (*RAD9-MYC/RAD9-HA* and *rad9ΔBRCTs-MYC/rad9ΔBRCTs-HA*, respectively), were treated with DNA damage and tested for the state of dimerization by Western blot analysis.

Despite several different approaches and numerous attempts, I have not been able to obtain clear co-immunoprecipitation data. Rad9 is incredibly sensitive to protein degradation upon cell lysis and it is also not readily soluble, making it difficult to immunoprecipitate a high yield of full-length protein. Also, in our hands, the HA epitope produces a very weak signal by antibody detection, thus compounding co-immunoprecipitation problems further. Efforts are currently underway to utilize the high-affinity TAP epitope tag in place of the HA epitope in order to complete the co-immunoprecipitation experiments.

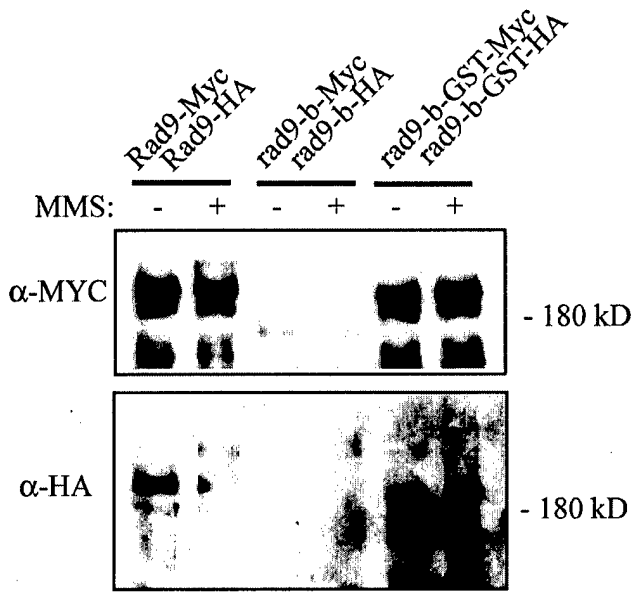

Figure 3-4. Co-immunoprecipitation of epitope-tagged Rad9 and rad9ΔBRCTs proteins. Diploid cells containing dually tagged Rad9 and rad9ΔBRCTs proteins expressed under control of the normal promoter were grown to the same cell density in untreated liquid media or media containing 0.1% MMS to induce DNA damage. Cells were then lysed and immunoprecipitated with α-MYC sepharose beads. Immunoprecipitated extracts were separated on a 6% acrylamide gel, transferred to a nitrocellulose membrane, and probed with α-Myc and α-HA antibodies. The full-length and truncated Rad9 proteins run slightly higher than the 180 kD marker indicated to the right of the Western blots.

The results shown in Figure 3-4 demonstrate the Western detection signals that I am able to attain. By pulling down Myc-tagged Rad9 or rad9ΔBRCTs fragments, I am able to co-immunoprecipitate the corresponding HA-tagged protein in both the wild-type strain containing full-length Rad9 and the in the strain containing the GST-tagged rad9ΔBRCTs fragment under both non-DNA-damaging and DNA-damaging conditions. No HA-tagged rad9ΔBRCTs appears in the co-immunoprecipitated Western blot for the rad9ΔBRCTs protein that lacks the GST fusion. While this is likely due to the inability of the protein to dimerize, such a statement cannot be definitively made since the protein levels present in the α-Myc Western blot are so low. If any co-immunoprecipitated HA-protein is present in the α-HA blot, it is probably undetectable by my methods given the low protein abundance. Though these blots are far from publishable, I am convinced by the data presented therein - namely that wild-type Rad9 dimerizes with and without DNA damage induction and that the rad9ΔBRCTs-GST protein is constitutively dimerized, as

expected. Publishable co-immunoprecipitation results that would directly confirm that the GST protein restores dimerization to the radΔBRCTs protein are still required.

Determining if Rad9 is constitutively dimerized irrespective of DNA damage.

The observation that Rad9 readily dimerizes irrespective of DNA damage is contrary to a previous hypothesis. Soulier & Lowndes (1999) showed that a fraction of Rad9 interacts with itself in undamaged cells, but that after damage induction, Rad9 molecules demonstrated a greater affinity for dimerization. Furthermore, this dimerization was shown to be dependent on the BRCT domains, as it was abrogated in a strain with point mutation of highly conserved aromatic residues within these domains. Because the data depicted in Figure 3-4 was obtained using strains with the temperature-sensitive *cdc13-1* mutation in the background, it is formally possible that these cells undergo a low level of endogenous damage at telomeres even though they are grown at the permissive temperature. The possibility of low levels of *cdc13-1* damage may account for the Rad9 dimerization I see when cells are not treated with MMS damage. To confirm if Rad9 does indeed constitutively dimerize regardless of DNA damage presence, I have created and introduced a *CDC13*$^+$ centromeric plasmid into the strains assayed in Figure 3-4 to eliminate any possible endogenous *cdc13-1* damage. Based on my crude results, it does indeed appear that Rad9 is constitutively dimerized irrespective of NDA damage (Figure 3-5). Upon better co-immunoprecipitation methods, these strains will be better assayed for dimerization with and without MMS treatment in the presence of *CDC13*$^+$.

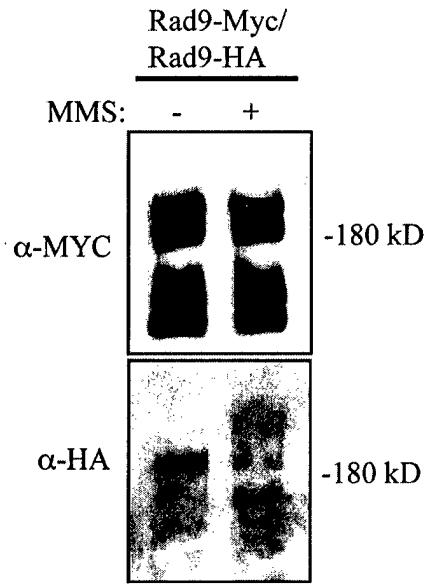

Figure 3-5. Co-immunoprecipitation of epitope-tagged Rad9 in a strain containing *CDC13*. Diploid cells containing dually tagged Rad9 and a *CDC13*$^+$ plasmid were grown in liquid media. Cells were either left untreated or treated with 0.1% MMS to induce DNA damage. Cells were then treated as described in Figure 3-4.

Activation of downstream kinases by rad9ΔBRCTs-GST.

Dimerization of Rad9, whether via the BRCTs or the GST protein, correlated with its phosphorylation in response to DNA damage, its activation and interaction with Rad53, and its activation of Chk1. The wild-type and rad9ΔBRCTs-GST proteins displayed hyperphosphorylation in response to *cdc13-1* and MMS-induced damage,

whereas the rad9ΔBRCTs-Myc construct was not substantially phosphorylated (Figure 3-6). Furthermore, phosphorylation of the dimerized proteins correlated with the ability of the proteins to interact with phosphorylated Rad53. That is, the rad9ΔBRCTs-GST protein was able to activate and co-immunoprecipitate phosphorylated Rad53 in the presence of *cdc13-1*- and MMS-induced damage as well as did wild-type Rad9, however

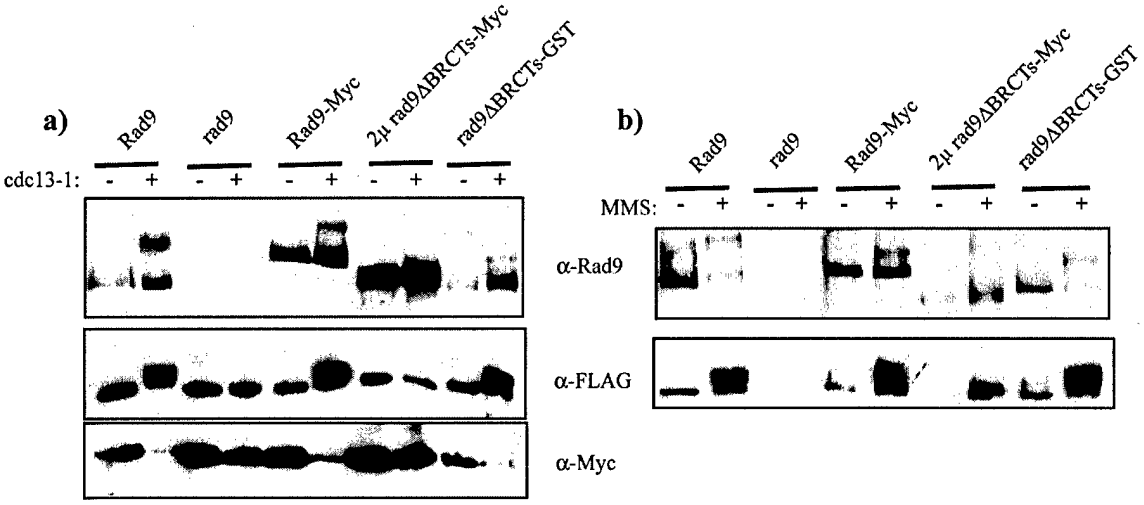

Figure 3-6. Activation of downstream kinases by dimerizing rad9ΔBRCTs-GST but not non-dimerizing rad9ΔBRCTs-Myc. (*a*) Total protein extracts were prepared by 20% TCA precipitation from cells either grown normally or subjected to *cdc13-1* damage for 3 hours at the restrictive temperature. (*b*) The top panel shows immunoprecipitation of Rad9 using α-Rad9 antibodies. The panel below shows co-immunoprecipitated Rad53-FLAG. TCA and IP Westerns were probed with either α-Rad9, α-FLAG antibody to detect Rad53-FLAG, or α-Myc to detect Chk1-Myc.

the non-dimerizing rad9ΔBRCTs-Myc protein was unable to do so (Figure 3-6b). Similarly, Chk1 was activated in both the wild-type and *rad9ΔBRCTs-GST* strain, whereas no Chk1 phosphorylation was evident in the non-dimerizing *rad9ΔBRCTs-MYC* strain (Figure 3-6a).

***rad9ΔBRCTs-GST* demonstrates cell cycle arrest kinetics identical to that of *RAD9*.**

Because the *rad9ΔBRCTs-GST* strain was virtually indistinguishable from the wild-type *RAD9* strain in the arrest and UV resistance assays, we sought to identify some difference in phenotype between the two strains, which might indicate some role of the BRCT domains above and beyond that of dimerization. In accord with the prevailing model, if Rad9 dimerizes only after DNA damage, we hypothesized that the constitutively-dimerized rad9ΔBRCTs-GST protein might render cells more responsive to DNA damage than wild-type cells by having already overcome the need to dimerize for function in the checkpoint cascade. To test this, cell cycle arrest was determined when cells were shifted to the semi-permissive temperatures of 28°C and 26.5°C to partially inactivate the temperature-sensitive cdc13 protein (the restrictive temperature for *cdc13-1*^{ts} strains is >28°C). No difference was seen between the percent of cells that arrested for either strain at either temperature (data not shown).

We also hypothesized that the *rad9ΔBRCTs-GST* strain may remain arrested for longer after damage insult if dissociation of the dimer was required to resume cell cycle progression, analogous to Rad9 dephosphorylation that is required for resuming cell cycle progression after damage (Pellicioli *et al.*, 2001; Esashi & Yanagida, 1999). Cells were thus incubated at the restrictive temperature to induce arrest and then shifted down to the permissive temperature to allow cells to resume cycling. Again, no difference was seen in the amount of *RAD9* and *rad9ΔBRCTs-GST* cells that arrested, and both strains resumed cell cycling by proceeding through mitosis at the same time and at the same rate (data not shown).

Both results – that *RAD9* and *rad9ΔBRCTs-GST* are similarly sensitive to DNA damage and that they show the same kinetics of cell cycle progression after DNA damage induction and repair - are consistent with possibility that Rad9 is actually constitutively dimerized, as the rad9ΔBRCTs-GST proteins are. Alternatively, it is possible that Rad9 dimerization is not rate-limiting for protein function. Regardless, these results lend credence to the ability of the dimerizing GST protein to completely restore rad9ΔBRCTs function by replacement of the BRCT domains and further support the hypothesis that the predominant role for the BRCTs in Rad9 is to mediate protein dimerization.

rad9ΔBRCTs-GST causes the same adaptation response as that of Rad9.

Cells constitutively assaulted with DNA damaging conditions arrest for about eight hours before undergoing adaptation, or resumption of cell cycle progression in the presence of DNA damage (Sandell & Zakian, 1993). This process appears to require the dephosphorylation of Rad9 to down-regulate the checkpoint cascade (Pellicioli *et al.*, 2001; Sanchez *et al.*, 1999), but whether the state of Rad9 dimerization must be altered for adaptation to occur is unknown. In accord with the model whereby Rad9 predominantly dimerizes upon damage induction, we sought to determine if dissociation of Rad9 dimers is required for adaptation, again, to discern a difference between *RAD9* and *rad9ΔBRCTs-GST* strains. *RAD9* and *rad9ΔBRCTs-GST* cells were incubated at the restrictive temperature, separated into individual large-budded cells on solid, rich media by micromanipulation, and maintained at the restrictive temperature for over eight hours. The individual large-budded cells were then analyzed for the presence of additional cell buds, indicative of adaptation. We found that 85.4% (41/48) of wild-type cells adapted and 83.3% (45/54) of *rad9ΔBRCTs-GST* cells adapted, thereby disfavoring the possibility that dissociation of Rad9 dimers is needed for adaptation. This point indirectly lends support to the notion that Rad9 may be constitutively dimerized, like rad9ΔBRCTs-GST, since no phenotypic difference was seen between the two strains analyzed. Alternatively, dissociation of Rad9 dimers may not be a key factor that determines the ability to adapt; perhaps, rather, the state of phosphorylation is a key determinant in the adaptation response, for which both proteins are similarly affected.

The role of Rad9 & rad9ΔBRCTs in response to S-phase damage.

While the role of Rad9 at the G_2/M cell cycle transition has been well characterized, its role during S-phase remains unclear. Whereas Rad9 is essential for a checkpoint response during G_2/M in the presence of DNA damage, *rad9* mutation causes only a weak increase in sensitivity of cells treated with MMS, an agent that produces DNA adduct addition causing wild-type cells to slow the rate of replication (Lopes et al.,

2001; Tercero & Diffley, 2001; Paulovich & Hartwell, 1995). Futhermore, *rad9* mutants demonstrate no appreciable sensitivity to hydroxyurea (HU), which depletes dNTP pools to slow the rate of S-phase replication. Still unresolved is whether Rad9 performs functions during S-phase that are different from those during G_2/M. While homodimerization via the BRCT domains may be required for specific Rad9 S-phase functions similar to those in G/M, such as interaction with Rad53, Rad9 could carry out additional S-phase roles by heterodimerizing with other proteins and/or by directly binding to DNA.

We used MMS to test the roles of our various deletion proteins in S-phase responses (Figure 3-7). These assays test both the requirement of the BRCT domains for Rad9 function in S-phase damaged cells and also the importance of Rad9 dimerization for these functions. The *RAD9-MYC, rad9ΔBRCTs-MYC,* and *rad9ΔBRCTs-GST* strains were treated with 0.033% MMS over time and assessed for cell sensitivity. As can be seen in Figure 3-7a, both the *RAD9-MYC* and *rad9ΔBRCTs-GST* strains are as MMS resistant as the wild-type *RAD9* strain. The *rad9ΔBRCTs-MYC* strain, on the other hand, is much more sensitive to MMS damage, though not as much as a *rad9* null strain, similar to its degree of UV sensitivity that we saw previously. This hypomorphic MMS resistance must be due to the inability of the rad9ΔBRCTs-MYC protein to homodimerize and not due to loss of some additional function mediated by the BRCT domains, such as heterodimeric interactions or DNA binding, since the homodimerizing *rad9ΔBRCTs-GST* strain also lacks the BRCT domains and the potential to heterodimerize or bind DNA but is wild-type for MMS resistance. Under the given conditions, these data indicate that in S-phase the BRCT domains are required for homodimerization to enable Rad9 function, implying that the role of Rad9 in S-phase is similar to its role in G_2/M.

Because Rad9 does not play a large role in the checkpoint response to damage during S-phase, it is hypothesized that other proteins function in the place of Rad9 in DNA damage recognition, processing, and/or repair during this phase of the cell cycle. Indeed, cells lacking Rad9 can be sensitized to MMS damage by elimination of either Tof1 or Mrc1, proteins thought to be partially redundant for Rad9 in mediating an S-phase checkpoint response to damage (Alcasabas et al., 2001; Foss, 2001). Hence, the *rad9 tof1* double mutant is as MMS sensitive as a *mec1* mutant, and the *rad9 mrc1* mutant is synthetically lethal.

To enhance the Rad9-dependent S-phase response to damage, the *TOF1* and *MRC1* genes were knocked out in our various Rad9-tagged strains. The strains were again subjected to MMS damage and analyzed for cell sensitivity. The addition of the *mrc1* mutation slightly sensitizes *RAD9*, *RAD9-MYC,* and *rad9ΔBRCTs-GST* strains to MMS damage compared to the wild-type *RAD9 MRC1* strain (Figure 3-7b). Whereas a *rad9 mrc1* strain is synthetically lethal, the *rad9ΔBRCTs-MYC mrc1* strain is viable but shows a more pronounced MMS sensitivity compared to the other strains tested. Likewise, addition of the *tof1* mutation has a similar, though lesser effect on cells (Figure 3-7c). *tof1* in combination with *RAD9, RAD9-MYC*, and *rad9ΔBRCTs-GST* is not appreciably sensitized to MMS damage, whereas the *rad9ΔBRCTs-MYC* strain show a greater sensitivity to MMS, though not as great as that of the *rad9 tof1* double mutant. These results indicate that the rad9ΔBRCTs-GST protein is fully functional for S-phase functions and that the roles of Rad9 in S-phase look to be equivalent to its roles in G_2/M.

Furthermore, because the *rad9ΔBRCTs-MYC* strain does retain some resistance to MMS damage, this suggests that some Rad9 function can be carried out without the function of the BRCT domains. What these functions entail remains to be determined.

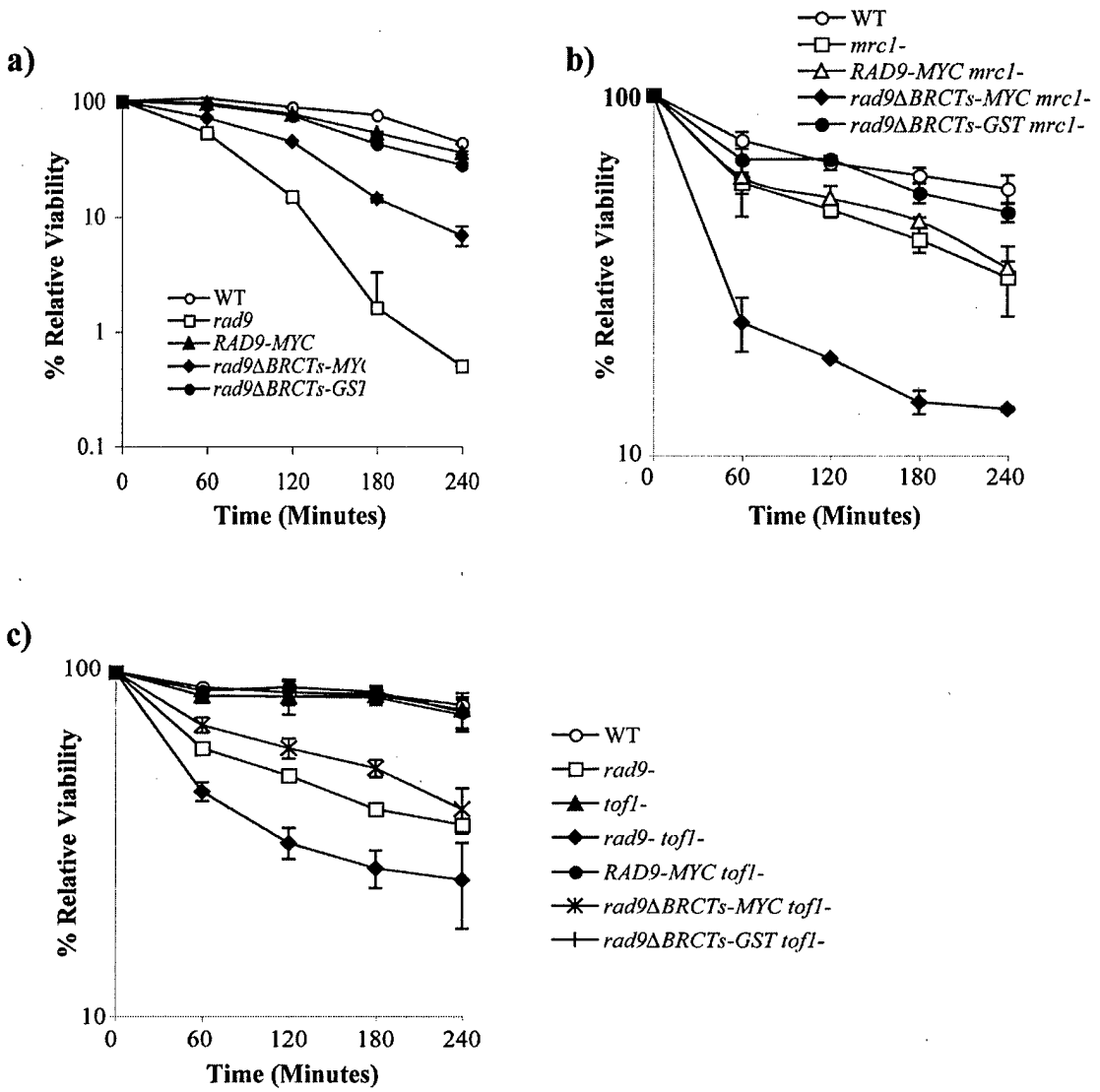

Figure 3-7. S-phase responses of cells containing non-dimerizing rad9ΔBRCTs-Myc and dimerizing rad9ΔBRCTs-GST. (*a*) Mid-logarithmic liquid cultures were treated with 0.033% MMS and plated to solid media at the times indicated. Cells were grown overnight at 23°C and scored the following day for microcolony formation. (*b*) MMS sensitivity of *mrc1*$^-$ strains was assayed as described in (*a*), except cells were treated with 0.00019% MMS. (*c*) MMS sensitivity of *tof1*$^-$ strains was assayed as described in (*b*). Duplicates of each strain were tested, and the average of the duplicates is shown above with error bars representing the standard deviation from the mean.

Analyzing *RAD9* BRCT point mutations and the effects on protein function.

The BRCT domains fold to form a globular structure with a hydrophobic core. Within each domain is an aromatic residue - either a phenylalanine or a tryptophan - that is the most highly conserved feature in BRCTs across all species (Bork et al., 1997; Callebaut et al., 1997). Based on crystal structure, this residue lies at the center of the highly conserved hydrophobic pocket (Zhang et al., 1998). Mutation of these aromatic residues is thought to severely abrogate proper BRCT domain folding. The Breast Cancer Information Core (BIC) database reports the mutation of the conserved tryptophan of the second BRCT motif of BRCA1 (W1708D) found in beast cancer tissue, indicating the severity of such a mutation (Soulier & Lowndes, 1999). Another reported mutation within the second BRCA1 BRCT domain, W1837R, also identified in breast cancer tissue, causes a hydrophobic residue to be replaced by charged residue that would presumably prevent proper formation of the hydrophobic fold (Zhang et al., 1998).

Point mutation of the highly conserved phenylalanine residue in Rad9's first BRCT domain (F1104A) and the tryptophan in the second BRCT domain (W1280A) were previously analyzed both singly (*rad^{MutF}* and *rad9^{MutW}*) and in combination (*rad9^{MutF+W}*; Soulier & Lowndes, 1999). It was shown that strains containing these mutated proteins are as sensitive to UV damage as a *rad9* null and also demonstrate cell cycle arrest defects in the presence of DNA damage. We also chose to assess the contribution of Rad9's BRCT domains using these point mutations. After confirming Soulier & Lowndes' initial results, we reasoned that Gal overexpression of the rad9^{MutF+W} point mutant might restore DNA damage and arrest functions as we found when we overexpressed the rad9ΔBRCTs protein under control of the Gal promoter. An overexpression construct containing the *rad9^{MutF+W}* sequence was created and overexpressed in a *rad9^{-}* background. Surprisingly, this strain was null in its ability to arrest in response to *cdc13-1* damage and in its ability to repair UV-induced DNA damage (Figure 3-8a, b). This result stands in contrast to that of overexpression of the rad9ΔBRCTs protein: rad9ΔBRCTs cannot dimerize via the BRCT domains presumably just like rad9^{MutF+W}, yet overexpression of rad9ΔBRCTs protein can rescue a *rad9^{-}* strain whereas overexpression of rad9^{MutF+W} cannot. This implies that the rad9^{MutF+W}-expressing strain is defective in some way that the rad9ΔBRCTs-expressing strain is not. Overexpression of the *rad9^{MutF+W}* construct in a *RAD9^{+}* background produced no dominant negative effect for either the ability to arrest or repair UV damage (data not shown).

A possible explanation for the previous result is that rad9^{MutF+W} still needs to dimerize for some reason in order to function. Given that the BRCT point mutations probably render Rad9 non-functional by abrogating dimerization, we predicted that fusion of the GST protein to the C-terminus of rad9^{MutF+W} might re-establish dimerization of the protein to restore its function, as it does for the rad9ΔBRCTs-GST protein. We tested arrest in response to damage and UV repair in these strains and found that normal expression (under control of the *RAD9* promoter) of rad9^{MutF+W}-GST, like both normal expression and overexpression of rad9^{MutF+W}, produced a null response (Figure 3-8c, d). Also, though the rad9^{MutF+W} protein was as abundant as wild-type Rad9, it did not undergo hyperphosphorylation as Rad9 did in response to DNA damage (Figure 3-8e). Moreover, the rad9^{MutF+W}-GST protein did not become hyperphosphorylated as rad9ΔBRCTs-GST did (Figure 3-6), though we are presumable restoring dimerization to

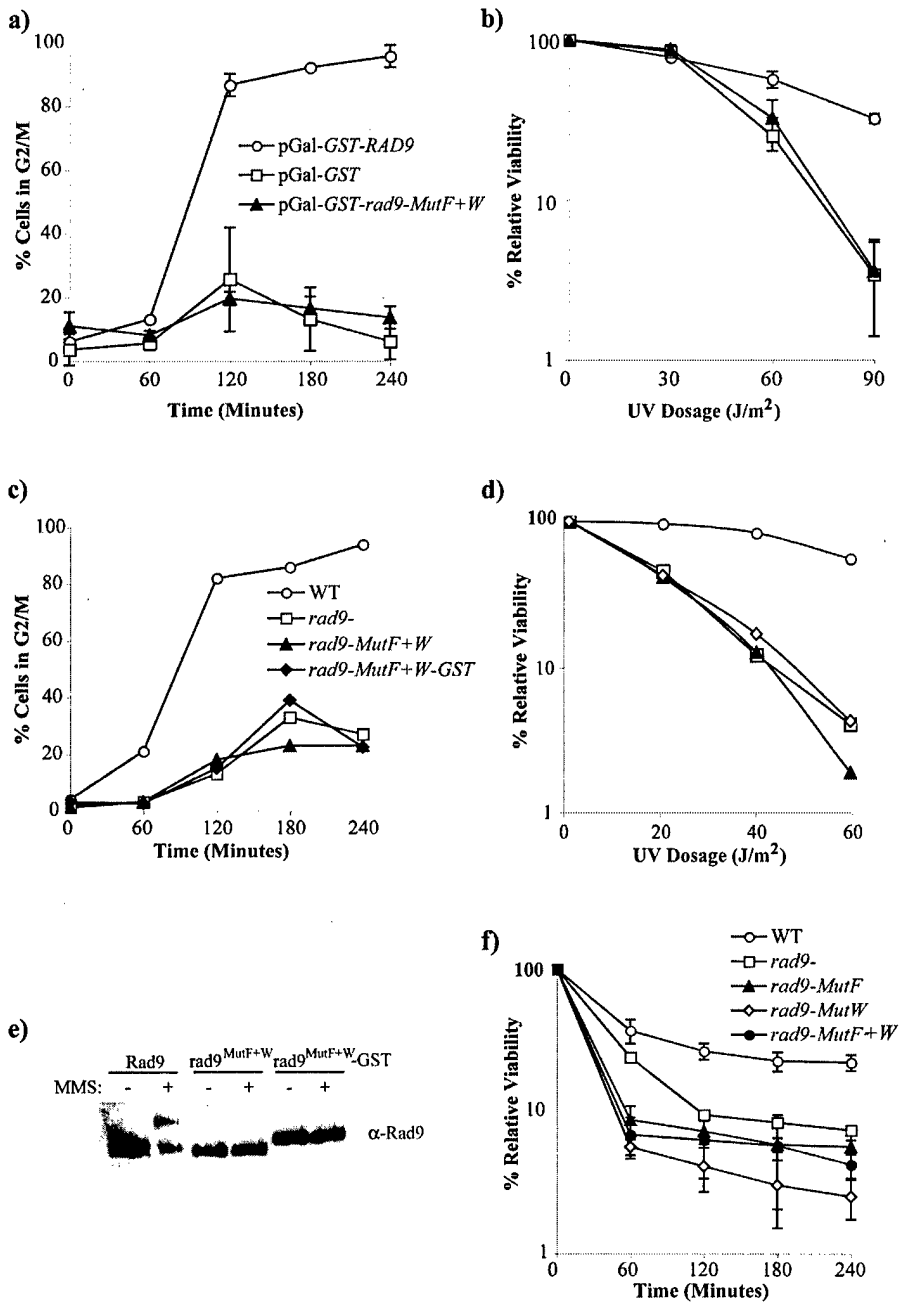

Figure 3-8. Phenotypic and protein characterization of cells containing rad9^{MutF+W} and rad9^{MutF+W}-GST. (*a*) Arrest and (*b*) UV sensitivity of *rad9* cells overexpressing the rad9^{MutF+W} protein. (*c*) Arrest and (*d*) UV sensitivity of *rad9^{MutF+W}-GST* cells under normal levels of expression. (*e*) Normal protein expression levels of rad9^{MutF+W} and rad9^{MutF+W}-GST proteins. (*f*) MMS sensitivity of *rad9^{MutF}*, *rad9^{MutW}*, and *rad9^{MutF+W}*. Assays were performed as previously described.

the rad9^{MutF+W} protein. We plan to examine whether the GST protein does indeed restore dimerization to rad9^{MutF+W} protein by co-immunoprecipitation analysis. We anticipate

that dimerization of rad9^{MutF+W}-GST proteins will be restored as it is for rad9ΔBRCTs-GST proteins. This result, then, would indicate that mutations within the rad9^{MutF+W} protein cause some dysfunction greater than that of merely abrogating Rad9 dimerization.

The rad9^{MutF}, rad9^{MutW}, and rad9^{MutF+W} proteins are all completely defective for S-phase functions.

To gain additional insight into Rad9 S-phase function, we determined the MMS sensitivity of each single BRCT mutant and the double *rad9^{MutF+W}* mutant. A recent study of the two BRCT domains in XRCC1 involving mutation of the same highly conserved aromatic residues as in the mutated *rad9^{MutF+W}* allele revealed that mutation of XRCC1 BRCT I rendered cells extremely MMS-sensitive, whereas mutation of BRCT II had little impact on the MMS resistance of cycling Chinese hamster ovary cells (Taylor et al., 2002). Such results suggest that BRCT I in XRCC1 may interact with a specific DNA repair protein to mend MMS-induced damage independent of BRCT II. Following this, we predicted that the single Rad9 BRCT point mutations might also demonstrate a different susceptibility to MMS-induced damage, indicative of different roles for each domain within S-phase. In contrast, we found that each BRCT single mutant was as MMS-sensitive as the double *rad9^{MutF+W}* mutant (Figure 3-8f), which was identical in sensitivity to that of the *rad9*$^-$ strain. This suggests that the two domains act in concert to mediate some S-phase function, and that mutation of one is sufficient to render the entire protein completely non-functional. The fact that the two single and the double BRCT point mutants are complete nulls for S-phase function stands in contrast to the phenotype of the *rad9ΔBRCTs-MYC* strain that lacks the BRCT domains altogether yet retains some S-phase function. This result, too, implies a greater protein dysfunction caused by point mutation of the BRCT domains.

Determining if the null phenotype of rad9^{MutF+W} can be explained by protein localization defects.

One simple explanation for the strange behavior of the rad9^{MutF+W} protein is that it is abrogated in its nuclear localization. The protein is expressed in abundances similar to that of wild-type Rad9, yet attempts to restore dimerization do not restore protein function. In contrast, the rad9ΔBRCTs-GST protein, which is expressed at abundances much less than that of Rad9, can fully complement Rad9 function. The rad9^{MutF+W} protein demonstrates no ability whatsoever to complement loss of Rad9 activity, whereas even the rad9ΔBRCTs-Mys protein that lacks the BRCT domains altogether demonstrates some UV and MMS-resistance. Even overexpression of the rad9^{MutF+W} protein has no effect on cell phenotype, while overexpression of rad9ΔBRCTs does. All these factors point to a defect that may simply be explained if the rad9^{MutF+W} protein is not correctly localized to the nucleus. Experiments are currently underway to test this possibility. If the rad9^{MutF+W} protein does show cellular localization defects, this may have implications for explaining the functional defects of BRCA1 proteins containing similar mutations.

Forcing aberrant Rad9 associations do not affect function.

To elucidate the conditions for Rad9 dimerization, we attempted to produce dominant-negative effects caused by aberrant dimerization using a variety of circumstances. The fact that the BRCT point mutations render overexpressed rad9^{MutF+W}

completely non-functional, whereas cells overexpressing rad9ΔBRCTs appear wild-type in response to damage, suggests that rad9^{MutF+W} is acting in an aberrant manner. To possibly account for the difference in phenotype between overexpressed rad9ΔBRCTs and normally expressed rad9^{MutF+W}, we reasoned that the BRCT domains might have a role in addition to that of dimerization by negatively regulating Rad9 *in cis* or *in trans*. By this model, in the case of the rad9ΔBRCTs deletion protein, the BRCTs are not present to inhibit protein function by negative regulation. Thus, when overexpressed, rad9ΔBRCTs can complement the null *rad9* phenotype by overcoming the need for dimerization by the BRCT domains and by not being negatively regulated by the BRCTs. For the rad9^{MutF+W} protein, however, the point mutations render the BRCT domains unable to dimerize, as was previously shown, and they yet may retain negative regulation. It is possible that dimerization is required to relieve this negative regulation (although some other protein modification, such as phosphorylation may also be required).

In trying to produce dominant negative effects, we tried several different conditions. We overexpressed the GST protein in the *rad9ΔBRCTs-GST* strain background and then induced DNA damage. We also overexpressed the truncated BRCTs and the BRCTs^{MutF+W} in the *rad9ΔBRCTs-GST* strain to see if this would produce a dominant negative effect based on a *cis* negative regulation hypothesis. Moreover, we attempted to force association of rad9ΔBRCTs-GST with the BRCTs by separately expressing GST-BRCTs and GST-BRCTs^{MutF+W} proteins in the *rad9ΔBRCTs-GST* strain background, while then inducing DNA damage to assess the resulting effects. Under all these conditions, we found no dominant negative effect. This may be explained if the affinity of rad9ΔBRCTs-GST dimerization is greater than the affinity for association with all the overexpressed fragments tested. A greater affinity of rad9ΔBRCTs-GST association with itself would be predicted if other regions of Rad9 aside from the BRCT domains promoted self-association in addition to the self-associating GST protein. Moreover, this same possibility – that other regions aside from the BRCT domains contribute some low-level affinity for Rad9 self-association – may also account for why we were never able to produce any dominant-negative effects in strains containing endogenous *RAD9*, assuming that Rad9 is constitutively dimerized as we predict it to be.

Does unphosphorylatable rad9 still dimerize?

Schwartz *et al.* (2002) recently identified several [S/T]/Q residues in Rad9 that undergo Mec1-dependent phosphorylation. Phosphorylation at seven of these sites in combination was shown to be critical for facilitating Rad53 interaction and activation, as the *rad9-7xA* strain did not activate Rad53 proteins nor could rad9-7xA co-immunoprecipitate Rad53 as wild-type Rad9 did. In contrast, the *rad9-7xA* strain was completely proficient for activation of another downstream kinase, Chk1, suggesting that phosphorylation of the residues necessary for Rad53 activation has no bearing on Chk1 activation. Consequently, *rad9-7xA* cells demonstrated the ability to partially arrest, since activation of Chk1 remained unaffected while Rad53 activation was abrogated.

While the previous study showed that phosphorylation of Rad9 is necessary for Rad53 activation, so, too, is dimerization thought to be important, though the ability of the rad9-7xA protein to dimerize via its BRCT domains was not previously assessed in this study. Homodimerization is thought to increase the local concentration of Rad9 such that two dimerized, hyperphosphorylated Rad9 molecules can interact with two Rad53

molecules, bringing them in close enough proximity with each other to allow for Rad53 autophosphorylation and, consequently, propagation of the checkpoint cascade (Gilbert *et al.*, 2001). While phosphorylation events needed to activate Rad53 do not affect Chk1 activation, dimerization of Rad9 might be important for Chk1 activation, as it is for Rad53.

Indeed, from my work, the rad9ΔBRCTs-Myc protein likely does not dimerize, and in this strain, Chk1 does not undergo phosphorylation in response to damage. In contrast, rad9ΔBRCTs-GST does dimerize, and Chk1 does get activated in this strain. This suggests, then, that dimerization of the Rad9 N-terminus is necessary for Chk1 phosphorylation in reponse to DNA damage. Consequently, we predict that the rad9-7xA protein should still be able to dimerize, since it can still activate Chk1.

If rad9-7xA can dimerize, this implies that the conditions for Rad9 dimerization are not dependent on its state of hyperphosphorylation by Mec1; the Rad9 phosphorylation event would appear to only affect Rad53 activation, as determined thus far. Moreover, such results would be consistent with the hypothesis that Rad9 is constitutively dimerized regardless of the presence of DNA damage. And if this were true, it would suggest that though Rad9 dimerization is necessary for Chk1 activation, it is not sufficient, or else the checkpoint arrest response would be constitutively partially activated. We presume some other Rad9 modification, aside from those that affect Rad53, or some additional protein interaction to be necessary to mediate Chk1 phosphorylation.

Though my data argue against it, if the rad9-7xA protein is unable to dimerize, this would indicate that the residues necessary for Rad53 interaction and activation are also necessary for dimerization. Furthermore, this would suggest that Rad9 dimerization (if it is not constitutive) is dependent on the state of phosphorylation, such that hypophosphorylated Rad9 molecules have a low affinity for dimerization while hyperphosphorylated molecules have a higher affinity for dimerization. This would then also lend insight into the mechanism of Chk1 activation, indicating that Chk1 activation is not dependent on the state of Rad9 dimerization.

To test the ability of rad9-7xA to dimerize by co-immunoprecipitation analysis, I created diploid cells containing rad9-7xA-Myc and rad9-7xA-HA proteins. These experiments have yet to be done due to our co-immunoprecipitation problems discussed previously.

KEY RESEARCH ACCOMPLISHMENTS

- endogenous expression of rad9ΔBRCTs results in a null phenotype, or no reponse to DNA damage, both in terms of UV resistance and cell cycle arrest
- overexpression of rad9ΔBRCTs produces a wild-type response to DNA damage both interms of UV resistance and cell cycle arrest
- restoring dimerization of Rad9 using the GST protein in place of the BRCT repeats (strain *rad9ΔBRCTs-GST*) restores a wild-type response to DNA damage both in terms of UV resistance and cell cycle arrest
- the dimerizing rad9ΔBRCTs protein is as active as wild-type Rad9 in that it becomes hyperphosphorylated after DNA damage and also interacts with and

induces hyperphosphorylation of the downstream checkpoint component Rad53 (also hyperphosphorylates the downstream kinase Chk1 like wild-type)
- the portion of Rad9 that interacts with Chk1 does not reside within the region encompassing the BRCT repeats, since restoring dimerization produces a wild-type arrest response
- Rad9 appears to be constitutively dimerized regardless of DNA damage presence (contrary to previous results of others) and the rad9ΔBRCTs-GST proteins also appear to constitutively dimerize
- the *rad9ΔBRCTs-GST* strain was identical in function to wild-type *RAD9* for all phenotypes tested including arrest kinetics, the adaptation response, and S-phase function in response to MMS damage (including when strains were additionally sensitized using *tof1* and *mrc1* mutations)
- expression of the BRCT repeats alone in a wild-type strain (*RAD9*$^{+}$) or in any of the mutant backgrounds (e.g., *rad9ΔBRCTs, rad9ΔBRCTs-GST*) does not produce a dominant negative effect
- expression of rad9ΔBRCTs-GST does not result in constitutive activation of the checkpoint response; this suggests that other events (e.g., phosphorylation by Mec1) are required in addition to dimerization to initiate a checkpoint response
- analysis of the *rad9*$^{MutF+W}$ strain shows phenotypes identical to that of the *rad9ΔBRCTs* strain, suggesting that any abrogation of the BRCT repeats impairs Rad9 function
- overexpression of rad9^{MutF+W} does not restore checkpoint functions as does overexpression of rad9ΔBRCTs, possibly suggesting that the BRCT domains play a role in negatively regulating Rad9 (the negative regulation is blocked from being relieved in the rad9^{MutF+W} mutated proteins)

REPORTABLE OUTCOMES

- research presented to fellow academics at my dissertation defense April 11, 2003 (Ph.D. awarded May 2003)
- abstract submitted and poster presented at the Era of Hope 2002 Department of Defense Breast Cancer Research Program Meeting, September 25-28, 2002
- poster presented at the 2002 Annual Biochemistry and Molecular & Cellular Biology Research Retreat, September 20-22, 2002
- abstract submitted and poster presented at the 2002 FASEB Summer Research Conference on Yeast Chromosome Structure, Replication, and Segragation, June28-July3, 2002
- oral presentation made at the 2001 Annual Biochemistry and Molecular & Cellular Biology Research Retreat, September 22, 2001

CONCLUSIONS

Rad9's BRCT domains predominantly function to concentrate Rad9.

My data indicates that, under normal levels of expression, the BRCT domains within Rad9 mediate homodimer interactions irrespective of DNA damage. Given the

phenotypes analyzed thus far, the predominant function of the BRCT domains appears to be to locally concentrate Rad9 by protein homodimerization, since deletion of both domains does not impair the arrest and repair responses of the rad9ΔBRCTs protein when it is overexpressed or when dimerization is restored using the GST protein. Additional Rad9 concentration can be achieved by protein stabilization, also mediated by the BRCT domains. Notably, the *rad9ΔBRCTs-GST* strain produces cell phenotypes identical to that of wild-type *RAD9* for the sensitivity to DNA damage, the kinetics of arrest in response to DNA damage, the kinetics of progression after DNA damage repair, and the extent of adaptation when persistent damage is present. In addition, the cell viabilities between the two strains are identical when cells are treated with either UV or MMS insult or when they are sensitized to MMS damage by addition of the *tof1* and *mrc1* mutations. This indicates that for these functions listed, the BRCT repeats predominantly act to locally concentrate Rad9 by homodimer formation and protein stabilization. Furthermore, since the rad9ΔBRCTs-GST protein is constitutively dimerized and acts identically to Rad9, this lends support to the idea that Rad9 is actually dimerized irrespective of DNA damage. Co-immunoprecipitation tests are underway to test this possibility and confirm that the rad9ΔBRCTs-GST proteins are constitutively dimerized.

Based on the results presented here, we can make certain predictions as to how Rad9 functions. Rad9 homodimerization is thought to be necessary for Rad53 activation. Consequently, homodimerization likely acts to increase the local concentration of Rad9 such that two dimerized Rad9 molecules can interact with two Rad53 molecules, bringing them in close enough proximity with each other to allow for Rad53 autophosphorylation and, consequently, propagation of the checkpoint cascade. We predict that the state of Rad9 homodimerization is also necessary for Chk1 activation, since the non-dimerizing *rad9ΔBRCTs-MYC* strain demonstrates no Chk1 phosphorylation in response to DNA damage, whereas the dimerizing *rad9ΔBRCTs-GST* strain does. We theorize that overexpression of rad9ΔBRCTs is sufficient to rescue arrest and repair in a *rad9* null strain by presumably overcoming the need for local concentration by boosting protein concentrations within the cell enough to mediate Rad9 functions. Alternatively, local Rad9 concentration mediated by BRCT dimerization can be attained by supplanting the BRCTs with another dimerizing domain, such as the GST protein.

Other possible roles mediated by Rad9's BRCT domains.

We hypothesized that the BRCT domains may mediate some other function, such as heterodimer interaction or DNA binding based on reports of other BRCT-containing proteins. If these BRCT functions are operable in Rad9, we predict them to be redundant with other functions as they have not been identified with the assay conditions described herein. Instead, we find that the BRCT domains may play a role in promoting Rad9 protein stability, since those proteins lacking the BRCTs altogether – rad9ΔBRCTs-Myc and rad9ΔBRCTs-GST – are expressed at levels considerably lower under control of the *RAD9* promoter than is wild-type Rad9. Curiously, the low protein expression levels do not affect protein function. The rad9ΔBRCTs-GST protein, expressed at one-forth the amount of wild-type Rad9, is still completely proficient for all Rad9 functions. Stabilizing Rad9 proteins, however, is one means to increase protein concentrations within the cell, though other mechanisms are required to additionally locally concentrate Rad9 for function.

Another possible role for the Rad9 BRCT domains is indicated by the enigmatic phenotype of the *rad9^{MutF+W}* strain. The rad9^{MutF+W} protein is expressed at levels similar to wild-type Rad9 when under the control of the *RAD9* promoter, yet the protein is completely nonfunctional as determined by phenotypic assays. This stands in contrast to the rad9ΔBRCTs-Myc protein that is expressed at greatly reduced levels in the cell compared to wild-type Rad9, yet retains some UV and MMS-resistance function. Furthermore, attempts to restore rad9^{MutF+W} activity, whether by overexpression or GST fusion, were completely ineffective though such strategies strikingly restored wild-type activity when applied to the rad9ΔBRCTs protein. Explanations that may account for the puzzling rad9^{MutF+W} phenotype include protein localization defects or negative regulation defects attributed to mutation of the highly-conserved aromatic residues within each BRCT domain. Additional experiments to test these hypotheses are currently being pursued.

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